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13. SUPPLEMENTARY NOTES

14. ABSTRACT

HO consists of formation of ectopic bone within muscles, connective tissues and blood vessels and can cause loss of normal posture and mobility, chronic pain, prosthesis fitting problems, deep venous thrombosis and other problems. HO is induced by trauma, burns and invasive surgeries and is thus very common amongst our severely wounded service-members. HO has in fact emerged as the single most important barrier to functional activity and return-to-duty in recent studies by DOD researchers and physicians. Current treatments are not wholly effective, are fraught with complications and may actually trigger additional HO in certain circumstances. Clearly there is an urgent need to create new, effective, specific and easy-to-deliver therapies for HO. The formation of HO lesions closely resembles the process by which endochondral bones normally form and grow during prenatal and postnatal life. Because that process requires a steep drop in activity of nuclear retinoic acid receptors (RARs), we hypothesized that acute pharmacological re-activation of the RARs could block HO. In previous studies sponsored by the USAMRMC, we did in fact find that synthetic selective RAR agonists are potent inhibitors of surgery-induced HO in mice. One of the most effective RAR agonists we tested was R667 (Palovarotene) previously used in an FDA-approved Phase 2 trial for another disease. R667 is thus our lead compound at present and will be used in additional HO animal models in this project.

15. SUBJECT TERMS
Heterotopic ossification; progenitor cells; chondrocytes; endochondral bone; marrow; chondrogenesis; retinoid agonists; retinoid signaling; retinoid nuclear receptors

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1. INTRODUCTION

Heterotopic ossification (HO) consists of formation of ectopic bone, usually endochondral bone, within muscles and other tissues, and is triggered by severe trauma, burns, neural damage and protracted immobilization. HO is particularly insidious in amputees where it can cause major complications, including prosthesis fitting problems, pain, local inflammation and pressure ulcer formation. HO has thus emerged as the most important barrier to restoration of functional activity and return-to-duty in recent studies of wounded active duty service-members conducted by this and other research groups. Because HO can be triggered by trauma, it can occur in patients in the general population undergoing invasive surgeries such as total hip arthroplasty. There is also a congenital form of it that can affect children and young adults. Surgery is often used to remove the HO lesions, but this procedure can have complications, may actually trigger another round of HO, and is not recommended for patients with recurrent HO and those suffering from congenital forms of it. Clearly, there is an urgent need to create new, effective and easy-to-deliver therapies for HO that could pave the way for a return to productive and functional life for amputees, trauma patients and other affected individuals. The formation of HO tissue masses starts with the recruitment of progenitor cells and their differentiation into chondrocytes and cartilage tissue. In our studies, we targeted this step and used retinoid agonists to block it in mouse models of HO. We found that several such drugs were effective, but agonists activating the nuclear retinoic acid receptor gamma (RARγ) were particularly effective. The purpose of the current project at our Institution is to determine whether the RARγ agonists also prevent HO in larger animals and whether side effects previously seen in fracture repair are reversed over time. The scope of the research is to study HO in diverse animals including rats and rabbits, analyze intramuscular and subdermal forms of HO, and examine rebound effects. The purpose of the research carried out by our collaborators at their Institution (Naval Medical Research Center) is to study the effectiveness of the drug treatment in a rat blast model of HO.

2. KEYWORDS

Heterotopic ossification; trauma; invasive surgery; congenital heterotopic ossification; progenitor cells; chondrogenesis; chondrocytes; endochondral bone; marrow; retinoid agonists; retinoid signaling; retinoid nuclear receptors

3. OVERALL PROJECT SUMMARY

This project addresses Heterotopic Ossification. HO consists of formation of ectopic bone, usually endochondral bone, within muscles, connective tissues and the blood vessel wall (1-6). HO is caused by trauma, burns, neural damage and other insults and can occur in patients undergoing invasive surgeries such as total hip arthroplasty (6). However, HO is more common in our wounded service-members than it would otherwise be expected on a pure statistical basis (7). This is likely due to the severity and encompassing nature of wounds and tissue damage suffered by the soldiers in war theaters and other conflicts. Daily function of HO patients is hampered by loss of normal posture, pain, prosthesis fitting problems, reduced mobility, formation of pressure ulcers, deep venous thrombosis or other complications (8-10). In fact, HO has emerged as the single most important barrier to functional activity and return-to-duty in a recent analysis of wounded active duty service-members (11). Current pharmacologic treatments are not wholly effective, have side effects that reduce patient compliance, and are not specifically directed against the skeletogenic process (12,13). Surgery is also often used to remove the HO lesions, but the procedure is fraught with complications, may actually trigger additional HO, and is not recommended for those in the general population suffering from congenital forms of it (14,15). Clearly, there is an urgent need to create new, effective, specific and easy-to-deliver therapies for HO, a debilitating disease that is hampering the return to productive life and service.

HO closely resembles the process by which endochondral bones normally form and grow during prenatal and postnatal life (14,16).This process initiates with recruitment and differentiation of progenitor cells into chondrocytes (17), a step that requires a steep drop in activity of nuclear retinoic acid receptors -RAR alpha (RARα), RAR beta (RARβ) and RAR gamma (RARγ)- (18-21). Thus, we hypothesized that acute pharmacological re-activation of the RARs (22,23) could inhibit chondrogenic cell differentiation and in turn prevent HO. To test this hypothesis, we carried out studies sponsored by the USAMRMC in which we used mouse models of HO mimicking surgery-induced subcutaneous or intramuscular HO (24). As we reported previously, we found that synthetic selective agonists for RARα or RARγ block these forms of HO, with the RARγ agonists being superior in terms of effectiveness (25,26). The drugs had no major side effects on overall systemic physiology except for a transient delay in bone fracture healing. Notably, the drugs also inhibited HO in a mouse model of Fibrodysplasia Ossificans Progressiva (FOP), a congenital, severe and often fatal form of HO (27). One of the most effective RARγ agonists we used was R667 (Palovarotene), previously tested for long-term treatment of a chronic disease (emphysema) in a FDA-approved Phase 2 trial (28). Thus, this is a case of drug repurposing, and R667 was and is our lead compound.

The objectives of the current CDMRP project are to establish the general validity of a retinoid agonist-based therapy against HO by testing additional animal models, gain insights into dose effectiveness and regimens, and verify safety. In this first year of funding, we have made progress at multiple levels toward these goals.

Given that the research goals of the project include new animal models and new experimental strategies, our first task during the initial few months of funding were to undergo

training in rat and rabbit use, care and testing. Researchers undergoing this training at that time were Drs. M. Pacifici, M. Iwamoto and K. Uchibe. Based on such training and information, we designed and wrote the appropriate animal protocols and submitted them to our IACUC. Once reviewed, the protocols were revised according to several issues and questions raised by the review committee members, and were submitted for further review. Protocols were eventually approved, and were also submitted to ACURO for their review and approval.

The project involves a close cooperation and interactions between our group at the Children's Hospital of Philadelphia and the research group led by Dr. Jonathan Forsberg at the Naval Medical Research Center (NMRC) in Bethesda, MD. Each group is responsible for distinct portions of the project that involve different experimental plans and animal protocols. Thus, this required the establishment of a Navy Cooperative Research and Development Agreement (NCRADA). The establishment and negotiation of the NCRADA involved also the participation of the Geneva Foundation located in Tacoma, WA that was going to be in charge of administering the funds allocated to the NMRC for the project. The NCRADA was completed by: identifying and describing the major research tasks and subtasks for each research group; providing details of respective experimental design and analytical methods; and describing in details the animal use and care protocols to be used by each group and the Institutional procedures followed to insure compliance and approval by both IACUC and ACURO. In addition, the expertise, experience and responsibilities of each research participant were provided that would be needed to fulfill and reach the goals of the overall project. The NCRADA was finalized in April of this year.

To review protocols, strategies and goals and harmonize them between the two research sites, we met with Dr. Forsberg and members of his team (Drs. T. Davis and L. Rosenbaum) here in Philadelphia. The discussion centered around the immediate steps to organize the research and the teams at the two locations, timetables for initiating animal studies, and lines of communication. Two of our team members (Drs. M. Iwamoto and K. Uchibe) subsequently traveled to Dr. Forsberg's laboratory in DC to compare first hand animal procedures, treatment modalities and analyses of outcomes. This interaction and subsequent continuous communications have been particularly important since we will be using subcutaneous and muscle-injury models of HO, while Dr. Forsberg's group will be using a blast model of HO.

The experimental work to be carried out by our group requires diverse technical skills including: ability to handle and perform surgery on experimental animal models; knowledge of cellular, biochemical and molecular procedures such as tissue processing and RNA analysis; and expertise in imaging techniques for hard tissues including microCT. Because of its extensive nature, the work is also very time consuming and requires a major commitment of time and effort. To be performed in a reliable and careful manner, the work requires researcher scientists with broad intellectual knowledge, good scientific judgment and critical thinking. Thus, we advertised a position for a postdoc fellow, interviewed candidates and were fortunate to be able to hire an excellent new postdoc –Dr. Sayantani Sinha- who started on January 21, 2014. During her PhD thesis work, Dr. Sinha was involved in vascular biology and pathology using mouse microsurgery, electrophysiology, molecular and cellular approaches, and in vivo and in vitro imaging. Thus, Dr. Sinha possesses all the technical and intellectual resources needed, in addition to a strong desire and commitment to expand her biomedical research horizon and expertise by entering musculoskeletal research in our project.

After joining us, Dr. Sinha has been working very closely with our senior postdoc Dr. Uchibe and our co-Investigator Dr. M. Iwamoto to gain expertise with HO animal

experimentation. Because the vast majority of our previous work was carried out in mouse, Dr. Uchibe used this animal species to train Dr. Sinha in the subcutaneous and intramuscular HO models. The first involves microsurgical implantation of a scaffold material (Matrigel) containing 1.0 μg of recombinant human BMP-2 serving as a skeletogenic agent. Though straightforward, this protocol is actually quite difficult since Matrigel quickly solidifies and needs to be handled with dexterity and without hesitation. This is challenging when 30 to 40 mice per experiment need to be implanted. In addition, the anatomical site of implantation needs to be exactly the same from mouse to mouse since extent and severity of HO formation are greatly influenced by surrounding tissues and in particular vasculature. This consistency allows for proper and effective comparisons of outcomes in control and treated animals. The intramuscular model is a trauma model, and involves microsurgical creation of a 2 mm pocket within the tibial muscles into which a collagen sponge pre-absorbed with 1 μg rhBMP-2 is inserted.

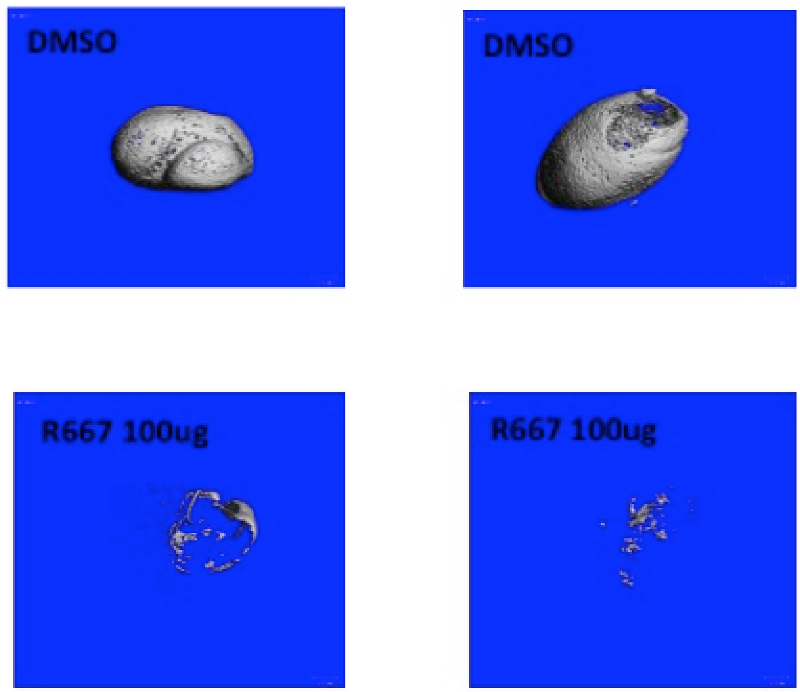

Figure 1. *Analysis of HO by μCT.* Top 2 panels show large round masses of HO present in control mice at day 14. Bottom 2 images show the significant reduction in HO in companion mice treated with R667 (100 μg/day).

This is a very challenging model since the pocket has to be created in a consistent and reproducible manner while avoiding excess bleeding, tissue damage and infections. Training Dr. Sinha using the mouse models first was also important since she could extend our previous findings and could also rely on our accumulated expertise and experience with these models, making predictions of results more reasonable and also solving unexpected complications in an easier and speedier manner. Over the last 6 months, Dr. Sinha has performed several sets of mouse experiments each carried out independently and each involving between 12 and 40 mice. This demanding schedule has allowed her to achieve significant microsurgical expertise and also knowledge of the different cellular, histological and molecular procedures and protocols for analyses of cartilage and bone required to assess and quantify effectiveness of drug treatment. The data she has obtained so far have shown that several retinoid agonists including R667 are effective against both subcutaneous and intramuscular forms of HO. Though early experiments elicited some variability from mouse to mouse in terms of both degree of HO in untreated mice and suppression of HO in treated mice, the last two sets of experiments have provided much more consistent observations. One example of these data is in Fig. 1. It shows μCT analysis of HO tissue formation in control mice receiving vehicle (corn oil) by gavage (top 2 panels) versus companion mice receiving R667 (100 μg/day) (bottom 2 panels). The data clearly show how effective the drug treatment was in reducing HO; image

analysis and quantification confirmed that the reduction exceeded 80%. They also show that consistency in surgical intervention, treatment modality and outcome analysis are optimal and effective.

Having attained this level of expertise with mice, Dr. Sinha (under supervision by Drs. Uchibe and Iwamoto) moved on to using one of the new animal models of HO and specifically rats. For these experiments, we used adult male Sprague-Dawley rats (350-400 gr) according to the new protocols approved by IACUC. Rats were anaesthetized by inhalation of isofluorane (4-5% for induction and 1-2% for maintenance) and injected subcutaneously with a 250-300 microliter mixture of matrigel and rhBMP2 at a prescribed abdominal site. In our previous mouse studies above, we had established that 1.0 to 1.25 μg of rhBMP2 in matrigel mixture was sufficient to elicit maximal HO. When tested in preliminary experiments in rats, however, this amount elicited minimal HO formation by 2 to 3 weeks. Thus, we performed new experiments and have now found that an amount of 2.5 to 3.0 μg in 250 μl matrigel is required, in agreement with a recent HO rat study (29).

After randomization, groups of 3 animals were implanted with the matrigel/rhBMP2

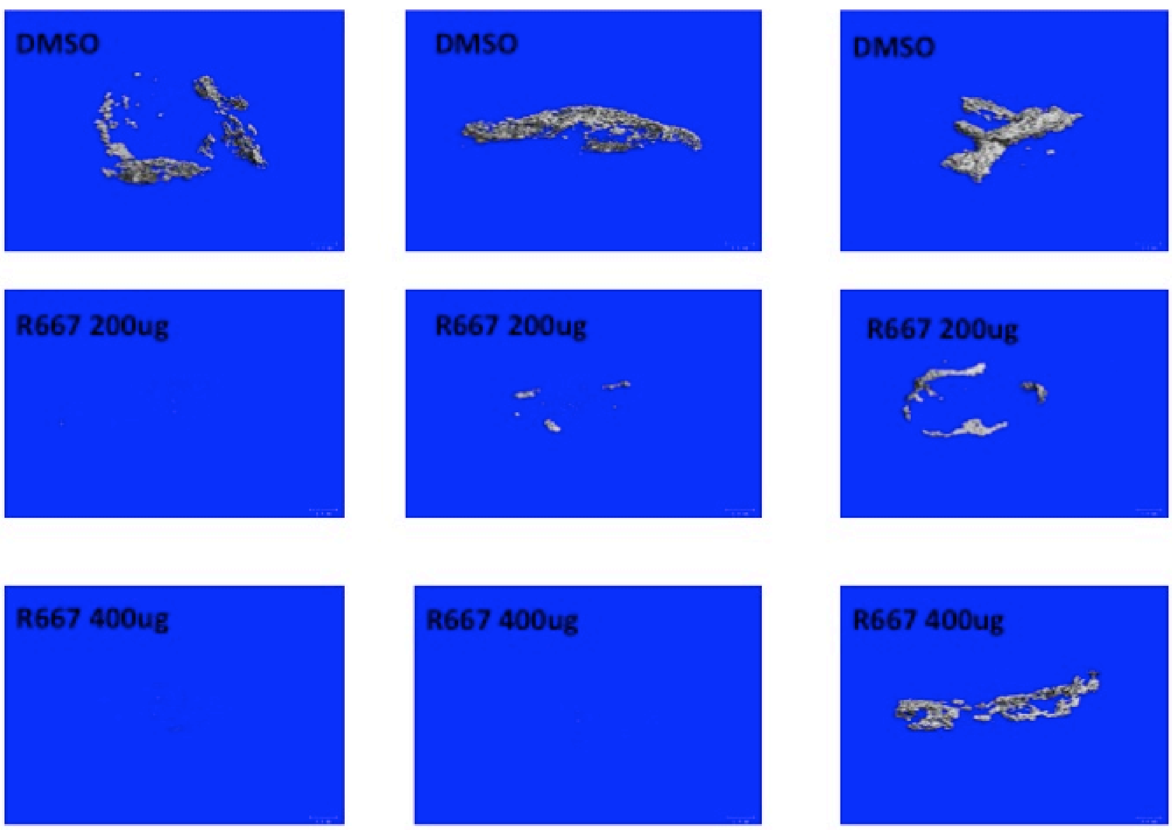

Figure 2. *HO rat model.* Rats (3 per group) were implanted with matrigel/rhBMP2 mixture subcutaneously and treated with vehicle (top) or 200 or 400 μg/day of R667 (middle and bottom panels). Note the significant reduction in HO in treated animals. See text for details.

mixture above and received a dose of 200 or 400 μg R667 by gavage (30) starting from day 1 from implantation. Treatment continued for 14 days. Parallel control animals received a daily dose of vehicle (10% DMSO in corn oil). The number of rats in each group was maintained low

in these experiments since they were our initial experiments. Because the differences between control and treated animals were expected to be high, the significance of the results was expected to be high, however. Ectopic tissue masses were harvested and subjected to imaging by μCT (Fig. 2) followed by histology (Fig. 3). The data in Fig. 2 indicate that a substantial amount of HO had developed in each of the 3 control rats, indicating that a consistent amount of matrigel/rhBMP-2 mixture had been implanted per animal and that the prescribed site of implantation elicited a similar degree of ectopic bone formation (Fig. 2, top 3 panels). Both doses of R667 had elicited a significant decrease in HO formation (Fig. 2, middle and bottom panels). However, effectiveness varied from animal to animal, with some animal exhibiting total or nearly total suppression and some animal showing partial inhibition. Because such variability was seen in both groups receiving 200 μg/day or 400 μg/day R667, the variability may have resulted from reasons rather than drug dose. We do not have a definitive explanation for the variability, but we can consider possibilities. As indicated above, the degree of HO formation depends on the efficiency of progenitor cell recruitment, rate and extend of chondrogenesis and cartilage maturation, and proximity and levels of neighboring vasculature. In previous mouse work, we found that the latter was a substantial variable given that osteoprogenitor cells are brought into ossifying sites by the vasculature itself. It is therefore possible that the variability observed in these initial rat experiments may derive from varying proximity of the implanted matrigel/rhBMP-2 mixture to subdermal vasculature, an issue we will address in future experiments. Another possibility is that drug delivery may have varied somewhat from rat to rat. Because these are our initial experiments with this animal model and because rats are more cumbersome to handle than mice, there may have been some variability in dispending drug by gavage. Though seemingly straightforward, gavage is actually not an easy technique and requires a considerable amount of time to master in a new species. The gavage needle itself has to be chosen carefully in terms of design and even if ideal, it can always elicit mechanical damage to the epithelia. In future experiments, we will sort out all these possibilities and will aim to minimize them, rendering the outcomes as consistent as possible. First, we will make sure that the matrigel/rhBMP-2 mixture (normally quite viscous) is maintained at low temperature throughout the injection procedure to keep it as fluid and homogenous as possible. Second, the anatomical site of implantation will be carefully preselected and marked in each animal. Third, the gavage drug delivery procedure will be reviewed for mechanics and organization, thus insuring that each animal is handled efficiently and similarly and that the gavage needle is inserted properly without any epithelial damage or scratch. The latter will be verified by collecting esophagus and trachea from the rats once sacrificed at the end of the experiment. The drugs are quite stable but are not water-soluble. Thus, we will also make sure that they are each solubilized well and homogenously before delivery. We believe all these measures will address and solve the rat-to-rat variability observed in the above initial experiments. Additional changes will be implemented should they be necessary.

Histologically, the ectopic HO masses displayed characteristic organization and structure. In HO in control rats, it was apparent that a significant amount of cartilage was present and was undergoing maturation and hypertrophy (Fig. 3, top panel). Because the HO samples had been harvested at day 14, they also displayed a sizable amount of endochondral bone and marrow (Fig. 3, top panel) that had elicited the conspicuous images seen by μCT (see Fig. 2). This overall tissue organization confirmed also that the process of ossification occurring in this model was in fact endochondral and that most likely, there was minimal concurrent intramembranous ossification. Histological images of ectopic masses from R667-treated rats showed that there was minimal amount of cartilage and endochondral ossification, and much of the masses embedded into the scaffold (matrigel) were largely composed of fibroblastic cells and connective tissues (Fig. 3, middle and bottom images).

Taken together, these initial experiments in the rat model are quite encouraging. They show that our team is rapidly acquiring expertise and proficiency, and our plan is to clarify the basis of some variability from animal to animal and minimize it as much as possible. This would not only render our observations more solid and reproducible but, as importantly, it should allow us to use fewer animals per experiments, thus permitting a greater number of overall experiments while maintaining the same overall number of animals expected to be used in the project. The data are encouraging because they show that the retinoid agonist strategy to prevent HO works well in rats as well. This conclusion will of course require many more experiments to be fully verified. It will also require comparison of effectiveness of at least an additional retinoid to be able to establish or at least strongly suggest, a drug class action.

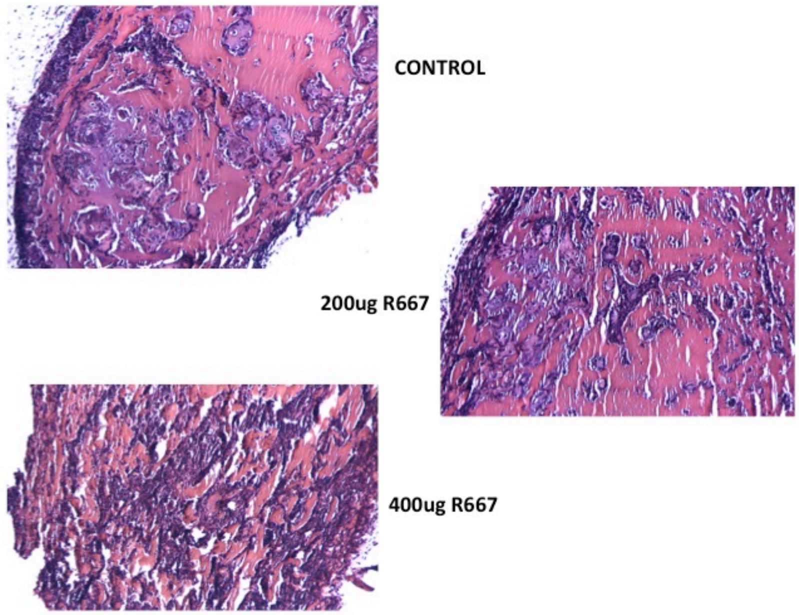

Figure 3. *Histological analysis of HO*. Ectopic tissue masses present on day 14 in control (top) and treated rats (middle and bottom) were processed for decalcification and standard paraffin histology using H&E staining. See text for details.

4. KEY RESEARCH ACCOMPLISHMENTS

-Establish and obtain approval of new rat and rabbit HO protocols from IACUC
-Submit and receive approval of those protocols by ACURO
-Establish a NCRADA between our Institution and the NMRC
-Organize meetings between the two research groups here and in Bethesda to review plans, share information on research protocols and procedure, coordinate work at respective sites, and establish lines of communication and coordination
-Recruit and train new postdoc personnel
-Perform in vivo experiments to establish effectiveness and reproducibility of HO animal model and insure expertise
-Carry out sets of preliminary experiments in the new animal model –rat-
-Document effectiveness of drug treatment by histological and imaging procedures
-Present data and accomplishments at several national and international symposia and workshops

5. CONCLUSON

The data obtained in the initial experiments with the rat model clearly suggest that the retinoid agonist treatment is quite effective in preventing HO. This provides a major and significant basis

to the project, re-affirming potency of this drug treatment. We can think of no other current pharmacological treatment that could elicit the same degree of HO prevention at present. In the coming year, we plan to extend these experiments to make sure the data are as solid and reproducible as possible. We need to compare at least two drugs to make sure they are both effective, thus establishing a drug class effect (that could be useful in the future should different drugs be needed). We need to carry out analyses to test whether there are side effects and whether these are reversible and minimal.

6. PUBLICATIONS, ABSTRACTS, AND PRESENTATIONS

a.

(1) Our research has been publicized by our Institution for a lay audience at the following web sites:

http://www.research.chop.edu/blog/experts-research-leads-rare-disease-clinical-trial/

http://www.research.chop.edu/btob/breakthrough-work-leads-to-rare-bone-disease-trial/

b. List of presentations:

(1) “Signaling pathways regulating chondrocyte function and heterotopic ossification”. Montefiore-Einstein University Medical Center, New York City, NY, October 2013

(2) “FOP therapy: steps ahead and implications for the treatment of related diseases”. IFOPA Association, Bologna, Italy, April 2014

(3) “Drug repurposing for the treatment of heterotopic ossification and Fibrodysplasia Ossificans Progressiva”. Musculoskeletal and Engineering Gordon Conference, Andover, NH, August 2014

(4) “Pharmacological prevention of heterotopic ossification”. Annual meeting of the American Society for Bone and mineral Research, Houston, TX, September 2014.

7. INVENTIONS, PATENTS AND LICENSES

Nothing to report

8. REPORTABLE OUTCOMES

Nothing to report

9. OTHER ACHIEVEMENTS

Nothing to report

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11. APPENDICES

Nothing to report